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Item No.: 3176

**FINAL DECISION DOCUMENT:
TSCA SECTION 5(H)(4) EXEMPTION FOR
BACILLUS SUBTILIS**

I. BACKGROUND

In the September 1, 1994, Federal Register (59 FR 45526), the Environmental Protection Agency (EPA) proposed at 40 CFR Part 700 under section 5(h)(4) of the Toxic Substances Control Act (TSCA), Tier I and Tier II exemptions. These exemptions, which would be found at § 725.400, are exemptions from EPA review and expedited EPA review, respectively, for certain microorganisms under certain use conditions. EPA proposed to include Bacillus subtilis at § 725.420 as a candidate recipient microorganism for the tiered exemptions. Bacillus subtilis is an ubiquitous bacterium commonly recovered from water, soil, air, and decomposing plant residue. B. subtilis produces a variety of proteases and other enzymes that enable it to degrade a variety of natural substrates and contribute to nutrient cycling. B. subtilis is one of the widely used microorganisms for the industrial production of enzymes and specialty chemicals.

This final decision document describes the basis for EPA's decision to include Bacillus subtilis as a recipient microorganism at § 725.420.

II. CONDITIONS OF EXEMPTION

EPA recognizes that some microorganisms present a low risk when used under specific conditions at general commercial use. Therefore, EPA proposed to institute expedited regulatory processes for certain microorganisms under these specific conditions at the general commercial use stage. Microorganism uses that are exempt would meet criteria addressing: (1) performance based standards for minimizing the numbers of microorganisms emitted from the manufacturing facility; (2) the introduced genetic material; and (3) the recipient microorganism. Microorganisms that qualify for these exemptions, termed Tier I and Tier II, must meet a standard of no unreasonable risk in the exempted use.

To evaluate the potential for unreasonable risk to human health or the environment in developing these exemptions, EPA focuses primarily on the characteristics of the recipient microorganisms. If the recipient is shown to have little or no potential for adverse effects, introduced genetic material meeting the specified criteria would not likely significantly

increase potential for adverse effects. As further assurance that risks would be low, EPA is also specifying procedures for minimizing numbers of organisms emitted from the facility. When balanced against resource savings for society and expected product benefits, these exemptions will not present unreasonable risks.

A. Criteria for Minimizing Release from Manufacturing Facilities

The standards proposed for the Tier I exemption were the following: (1) the structure(s) be designed and operated to contain the microorganism, (2) access to the structure should be limited to essential personnel, (3) inactivation procedures shown to be effective in reducing the number of viable microorganisms in liquid and solid wastes should be followed prior to disposal of the wastes, (4) features to reduce microbial concentrations in aerosols and exhaust gases released from the structure should be in place, and (5) general worker hygiene and protection practices should be followed.

1. Definition of structure. EPA considers the term "structure" to refer to the building or vessel which effectively surrounds and encloses the microorganism. Vessels may have a variety of forms, e.g., cubic, ovoid, cylindrical, or spherical, and may be the fermentation vessel proper or part of the downstream product separation and purification line. All would perform the function of enclosing the microorganism. In general, the material used in the construction of such structure(s) would be impermeable, resistant to corrosion and easy to clean/sterilize. Seams, joints, fittings, associated process piping, fasteners and other similar elements would be sealed.

2. Standards to minimize microbial release. EPA is taking, for several reasons, a somewhat cautious approach in prescribing standards for minimizing the number of microorganisms emitted through the disposal of waste and the venting of gases. First, a wide range of behaviors can be displayed by microorganisms modified consistent with EPA's standards for the introduced genetic material. Second, EPA will not conduct any review whatsoever for Tier I exemptions. EPA believes the requirement to minimize emissions will provide a measure of risk reduction necessary for making a finding of no unreasonable risk. Taken together, EPA's standards ensure that the number of microorganisms emitted from the structure is minimized.

EPA's standards for minimizing emission specify that liquid and solid waste containing the microorganisms be treated to give a validated decrease in viable microbial populations so that at least 99.9999 percent of the organisms resulting from the fermentation will be killed. Since the microorganisms used in fermentation processes are usually debilitated, either

intentionally or through acclimation to industrial fermentation, the small fraction of microorganisms remaining viable after inactivation treatments will likely have a reduced ability to survive during disposal or in the environment. Moreover, industrial companies, in an attempt to keep their proprietary microorganisms from competitors and to reduce the microbial numbers to those permitted by local sanitation authorities, modify the microorganisms to increase the ability of their microorganisms to survive and perform their assigned tasks in the fermentor but decrease their ability to survive in the environment external to the fermentor.

EPA requirements also address microorganisms in the exhaust from the fermentor and along the production line. To address exhaust from fermentors, EPA is requiring that the number of microorganisms in fermentor gases be minimized by the use of standard industry equipment prior to the gases being exhausted from the fermentor. EPA selected this standard based on an estimate of the numbers of microorganisms likely to be in the exhaust from an uncontrolled fermentor and common industry practice. Moreover, microorganisms that are physiologically acclimated to the growth conditions within the fermentor are likely to be compromised in their ability to survive aerosolization. EPA anticipates, therefore, that few microorganisms will survive the stresses of aerosolization associated with being exhausted with the gases from the fermentor. The provision requiring reduction of microorganisms in fermentor exhaust gases contributes to minimizing the number of viable microorganisms emitted from the facility.

EPA is also requiring that other systems be in place to control dissemination of microorganisms by other routes. This would include programs to control pests such as insects or rats, since these might serve as vectors for carrying microorganisms out of the fermentation facilities.

3. Worker protection. The requirement to minimize microbial emissions, in conjunction with the requirement for general worker safety and hygiene procedures, also affords a measure of protection for workers. Potential effects on workers that exist with microorganisms in general (e.g., allergenicity) will be present with the microorganisms qualifying for this exemption. As with other substances that humans may react to (e.g., pollen, chemicals, dust), the type and degree of allergenic response is determined by the biology of the exposed individual. It is unlikely that a microorganism modified in keeping with EPA's specifications for the introduced genetic material would induce a heightened response. The general worker hygiene procedures specified by EPA should protect most individuals from the allergenic responses associated with microorganisms exhausted from fermentors and/or other substances emitted along the production line. The EPA requirement that

access to the structure be controlled also addresses this consideration by reducing to a minimum the number of individuals exposed.

4. Effect of containment criteria. As further assurance that risks would be low, EPA is specifying procedures for minimizing the number of organisms emitted from the facility for the Tier I exemption. EPA is not specifying standards for minimizing the number of microorganisms emitted from the facility for microorganisms qualifying for Tier II exemption. Rather, the Agency requests that submitters utilize as guidance the standards set forth for Tier I procedures. The procedures proposed by the submitter in a Tier II exemption request will be reviewed by the Agency. EPA will have the opportunity to evaluate whether the procedures the submitter intends to implement for reducing the number of organisms emitted from the facility are appropriate for that microorganism.

B. Introduced Genetic Material Criteria

In order to qualify for either the Tier I or Tier II exemption, any introduced genetic material must be limited in size, well characterized, free of certain nucleotide sequences, and poorly mobilizable.

1. Limited in size. Introduced genetic material must be limited in size to consist only of the following: (1) the structural gene(s) of interest; (2) the regulatory sequences permitting the expression of solely the gene(s) of interest; (3) the associated nucleotide sequences needed to move genetic material, including linkers, homopolymers, adaptors, transposons, insertion sequences, and restriction enzyme sites; (4) the nucleotide sequences needed for vector transfer; and (5) the nucleotide sequences needed for vector maintenance.

The limited in size criterion reduces risk by excluding the introduction into a recipient of extraneous and potentially uncharacterized genetic material. The requirement that the regulatory sequences permit the expression solely of the structural gene(s) of interest reduces risk by preventing expression of genes downstream of the inserted genetic material. The limitation on the vector sequences that are components of the introduced genetic material prevents the introduction of novel traits beyond those associated with the gene(s) of interest. The overall result of the limited in size criterion is improved ability to predict the behavior of the resulting microorganism.

2. Well characterized. For introduced genetic material, well characterized means that the following have been determined: (1) the function of all of the products expressed from the structural gene(s); (2) the function of sequences that participate in the regulation of expression of the structural

gene(s); and (3) the presence or absence of associated nucleotide sequences.

Well characterized includes knowledge of the function of the introduced sequences and the phenotypic expression associated with the introduced genetic material. Genetic material which has been examined at the restriction map or sequence level, but for which a function or phenotypic trait has not yet been ascribed, is not considered well characterized. Well characterized would include knowing whether multiple reading frames exist within the operon. This relates to whether more than one biological product might be encoded by a single sequence, and addresses the possibility that a modified microorganism could display unpredicted behavior should such multiple reading frames exist and their action not be anticipated.

3. Free of certain sequences. In addition to improving the ability to predict the behavior of the modified microorganism, the well characterized requirement ensures that segments encoding for either part or the whole of the toxins listed in the proposed regulatory text for the TSCA biotechnology rule would not inadvertently be introduced into the recipient microorganism.

These toxins are polypeptides of relatively high potency. Other types of toxins (e.g., modified amino acids, heterocyclic compounds, complex polysaccharides, glycoproteins, and peptides) are not listed for two reasons. First, their toxicity falls within the range of moderate to low. Second, these types of toxins generally arise from the activity of a number of genes in several metabolic pathways (multigenic).

In order for a microorganism to produce toxins of multigenic origin, a large number of different sequences would have to be introduced and appropriately expressed. It is unlikely that all of the genetic material necessary for producing multigenic toxins would be inadvertently introduced into a recipient microorganism when requirements that the genetic material be limited in size and well characterized are followed.

Similarly, other properties that might present risk concerns result from the interactive expression of a large number of genes. For example, pathogenic behavior is the result of a large number of genes being appropriately expressed. Because of the complex nature of behaviors such as pathogenicity, the probability is low that an insert consisting of well characterized, limited in size genetic material could transform the microorganisms listed for exemption into microorganisms which display pathogenic behavior.

4. Poorly mobilizable. Poorly mobilizable means the ability of the introduced genetic material to be transferred and

mobilized is inactivated, with a resulting frequency of transfer of less than 10^{-8} transfer events per recipient. The requirement that the introduced genetic material be poorly mobilizable reduces potential for transfer of introduced genetic sequences to other microorganisms in the environment. Such transfers would occur through the interaction of the introduced microorganism with indigenous microorganisms through conjugation, transduction, or transformation. Through such transfers, the introduced genetic material could be transferred to and propagated within different populations of microorganisms, including microorganisms which may never previously have been exposed to this genetic material. It is not possible to predict how the behavior of these potential recipient microorganisms will be affected after uptake and expression of the genetic material.

Since EPA is not limiting the type of organism that can serve as the source for the introduced genetic material, some limitation is placed on the ability of the introduced genetic material to be transferred. This limitation mitigates risk by significantly reducing the probability that the introduced genetic material would be transferred to and expressed by other microorganisms.

The 10^{-8} frequency is attainable given current techniques. Plasmids with transfer rates of 10^{-8} exist or are easily constructed. Some of the plasmids most commonly employed as vectors in genetic engineering (e.g., pBR325, pBR322) have mobilization/transfer frequencies of 10^{-8} or less.

The criteria set for "poorly mobilizable" for transduction and transformation should be readily met since the majority of transfer frequencies reported for transduction and natural transformation are less than 10^{-8} . Higher frequencies are likely only under special circumstances, such as when the introduced genetic material has been altered or selected to enhance frequency. Because the risk concern EPA addresses with the 10^{-8} criterion is spread of the introduced genetic material broadly through microbial populations, exchanges between very closely related microorganisms, even if occurring at high frequency, is not a concern so long as the spread through populations does not occur at high frequency.

Fungal gene transfer has also been considered in development of the poorly mobilizable criterion. Although mobile genetic elements such as transposons, plasmids and double stranded RNA exist in fungi and can be readily transferred, this transfer usually is only possible between members of the same species during anastomosis, a process specific to fungi. Since anastomosis only occurs between members of the same species, the introduced genetic material would not be transferred to distantly related fungi as may occur with bacteria.

5. Effect of introduced genetic material criteria. The requirements placed on the introduced genetic material, in concert with the level of safety associated with Bacillus subtilis, ensure that the resulting microorganisms present low or negligible risk. The probability is low that the insertion of genetic material meeting EPA's criteria into strains of B. subtilis will change their behavior so that they would acquire the potential for causing adverse effects. Risks would be mitigated by the four criteria placed on the introduced genetic material, the relative safety of B. subtilis, and the inactivation criteria specified for the Tier I exemption. In the case of Tier II exemption, risks would be mitigated in light of the four criteria placed on introduced genetic material, the relative safety of B. subtilis, and EPA's review of the containment conditions selected.

C. Recipient Microorganism Criteria

Six criteria were used by EPA to determine eligibility of recipient microorganisms for the tiered exemption. All of the criteria were used together to determine whether, on balance, the microorganisms would not present an unreasonable risk to human health or the environment. Microorganisms which EPA finds meet these criteria are listed as eligible recipients. The first criteria would require that it be possible to clearly identify and classify the microorganism. Available genotypic and phenotypic information should allow the microorganism to be assigned without confusion to an existing taxon which is easily recognized. Second, information should be available to evaluate the relationship of the microorganism to any other closely related microorganisms which have a potential for adverse effects on human health or the environment. Third, there should be a history of safe commercial use for the microorganism. Fourth, the commercial uses should indicate that the microorganism products might be subject to TSCA jurisdiction. Fifth, studies are available which indicate the potential for the microorganism to cause adverse effects on human health and the environment. Sixth, studies are available which indicate the survival characteristics of the microorganism in the environment.

After each microorganism was reviewed using the six evaluation criteria, a decision was made as to whether to place the microorganism on the list. The Agency's specific determination for Bacillus subtilis is discussed in the next unit.

III. EVALUATION OF BACILLUS SUBTILIS

A. History of Use

1. History of safe commercial use. Bacillus subtilis is one of the most widely used bacteria for the production of enzymes and specialty chemicals. Industrial applications include production of enzymes, antibiotics, and other specialty chemicals. B. subtilis is considered a Class 1 Containment Agent under the NIH Guidelines for Research Involving Recombinant DNA Molecules and falls under the Class 1 Containment under the European Federal of Biotechnology guidelines.

2. Products subject to TSCA jurisdiction. To date, EPA has reviewed three premanufacture notices (PMNs) for strains of B. subtilis. One of the strains was modified for enhanced production of the enzyme α -amylase to be used primarily in production of ethanol. Another strain was modified for enhanced production of a lipase enzyme for use in heavy duty detergents.

B. Identification of Microorganism

1. Classification. The genus Bacillus consists of a large number of diverse, rod-shaped gram positive bacteria which are capable of producing endospores that are resistant to adverse environmental conditions. B. subtilis is the type species of the genus. Historically, B. subtilis was a term given to all aerobic endospore-forming bacilli. Numerous species that appeared in the early literature as B. subtilis have since been designated as separate Bacillus species. B. subtilis can be distinguished from closely related Bacillus species by the use of API diagnostic test kits or pyrolysis gas-liquid chromatography. Because of changes in the classification of the genus and recent developments in methods of taxonomic identification, older strains may not actually be B. subtilis under present-day definitions.

2. Related taxa of concern. B. subtilis is part of the same large cluster of bacilli which includes pathogenic or opportunistic Bacillus species. This includes the B. cereus/anthracis/thuringiensis/mycoides group whose members are mammalian and insect pathogens and food poisoning agents. However, B. subtilis is distinguishable from the pathogenic bacilli as well as from the more closely related bacilli.

C. Risk Summary

1. Studies regarding potential for adverse effects. B. subtilis is not a frank human pathogen, but has been isolated

from human infections. However, the literature suggests that before infection can occur, there must be immunosuppression of the host followed by inoculation in high numbers of the microorganism. B. subtilis does not produce significant quantities of extracellular enzymes or toxins and is generally considered to have a low degree of virulence. B. subtilis does produce the extracellular enzyme subtilisin that has been reported to cause allergic or hypersensitivity reactions in individuals repeatedly exposed to it.

The literature also indicates that ecological hazards associated with the use of B. subtilis are low. While there are reports suggesting that B. subtilis is a cause of abortion in livestock, Koch's postulates have not been satisfied in demonstrating that this microorganism was the causal agent. The association of B. subtilis with livestock abortions is quite low compared to the total number of livestock abortions caused by microorganisms. B. subtilis is not considered a plant pathogen.

2. Studies regarding survival in the environment. B. subtilis is a ubiquitous soil microorganism that contributes to nutrient cycling when biologically active, due to the various enzymes produced by members of the species. Unless a soil has been recently amended with organic matter providing readily utilizable nutrients, the bacilli exist in the endospore stage.

IV. PUBLIC COMMENTS RELEVANT TO THE RISK ASSESSMENT

A request for comments was made in the Proposed Decision Document for B. subtilis regarding the recommendation that since the hazards associated with the use of B. subtilis in fermentation were low, all strains of B. subtilis should be included in the tiered exemption even though the Proposed Risk Assessment recommended only those strains with a sporulation deficiency of 10^{-7} . However, no comments were received on this specific microorganism either in general or addressing this request for comments. There were a number of comments received on the tiered exemption, however, and some of these comments are relevant to the criteria discussed at Section II.A. of this document considered in listing this microorganism as an eligible recipient microorganism at § 725.420.

Some of the general comments received on the exemptions addressed the six criteria EPA used to select candidate recipient microorganisms to include at § 725.420. None of these comments questioned the eligibility of B. subtilis for inclusion on the list at § 725.420. EPA's responses to comments on how it used the six criteria to select candidate microorganisms at § 725.420 are detailed in the Response to Comments document to be found in the docket for this rulemaking.

The other comments pertained to the proposed containment conditions at § 725.422. Commenters questioned two criteria, the first of which is at § 725.422(b): "limit entry only to those persons whose presence is critical to the reliability or safety of the activity". Commenters pointed out that under the requirement as proposed, managers may be precluded from allowing administrative personnel, customers, and school and other educational tours into the facility. EPA had not intended to constrain facility managers to this extent and reconsidered the standard. EPA has revised § 725.422(b) to read "Control access to the structure". Additional explanation for this revision is given in the Response to Comments document (Section III.C.4.a.) and the Preamble of the Final Rule. Both of these documents can be found in the docket for this rulemaking.

The other criterion in the proposed rule which commenters questioned was at § 725.422(e): "provide and document effectiveness of features to reduce microbial concentrations by at least two logs in aerosols and exhaust gases released from the structure". Commenters argued that the requirement as written would require retrofitting of equipment in order to permit measurement within the fermentor headspace of microbial concentrations in aerosols. EPA had not intended that manufacturers be required to retrofit their fermentation equipment in order to qualify for this exemption. Therefore, EPA re-examined the basis for this criterion by reviewing information submitted on physical containment and control technologies in the PMNs it had received for intergeneric microorganisms between 1986 and 1995. Examination of these PMNs revealed that the number of microorganisms potentially released through fermentor exhaust gases is negligible compared to the number contained in the liquid and solid waste streams. Even under a worst case scenario of an uncontrolled release, as evaluated in the accompanying risk assessment, the number of viable microorganisms aerosolized with the fermentor exhaust gases would still be low, and therefore, the risk would remain low. Moreover, the use of a criterion requiring controls to minimize microbial numbers released through aerosolization at § 725.422, as compared to the worst case scenario of an uncontrolled release, would result in lesser exposure, and therefore, lower risk than under the uncontrolled release scenario. Uncontrolled releases are not standard industry practice because there are a number of economic considerations driving the control of exhaust gases such as maintaining proper molality of the fermentation broth by the use of a vapor recovery system, maintaining sterility, and preventing release of microorganisms for proprietary reasons. Therefore, upon re-evaluation, the Agency decided that language requiring minimization of microbial concentrations in aerosols could be substituted for the requirement of the 2-log reduction performance criterion without affecting the no unreasonable risk finding necessary for a 5(h)(4) exemption under TSCA. The potentially increased exposure to this organism from the

modification of the containment criteria from the proposed 2-log reduction to minimizing microbial numbers in exhaust gases does not change the risk of using this microorganism for fermentation. Therefore, EPA has revised § 725.422(e) to read: "Use features known to be effective in minimizing viable microbial populations in aerosols and exhaust gases released from the structure, and document use of such features". The Response to Comments document (Section III.C.4.b.) and the Preamble of the Final Rule provide a thorough explanation for the change in requirements for microbial releases through exhaust gases.

V. BENEFITS SUMMARY

Substantial benefits are associated with this proposed exemption. Bacillus subtilis is already widely employed in general commercial uses, some of which are subject to TSCA reporting. The microorganism produces a variety of enzymes such as protease, amylase, and lipase which has wide applications in the detergent industry. Proteases are also used for dehairing and batting in the leather industry, and amylases for desizing of textiles and sizing of paper. In addition, the microorganism is capable of producing a wide variety of specialty chemicals and antibiotics, some of which are TSCA uses. The Agency believes this exemption will result in resource savings both to EPA and industry without compromising the level of risk management afforded by the full 90 day review. The exemption will result in reduced reporting costs and a decrease in delay associated with reporting requirements. The savings in Agency resources can be directed to reviewing activities and microorganisms which present greater uncertainty. This exemption should also facilitate development and manufacturing of new products and the accumulation of useful information.

VI. FINAL ANALYSIS

1. Risks from use of the recipient microorganism B. subtilis are low. B. subtilis is ubiquitous in the environment and the releases expected from fermentation facilities will not significantly increase populations of this microorganism in the environment. Although the possibility of human infection by B. subtilis is not non-existent, it is low in the industrial setting, because it occurs primarily in highly immunocompromised individuals. In the industrial setting with the use of proper safety precautions, good laboratory practices, and proper protective clothing and eyewear, the potential for adverse human health effects is quite low. Although B. subtilis may be associated with livestock abortions, the use of this microorganism in fermentation facilities will not substantially increase the frequency of this occurrence because it is widespread in the environment.

2. Use of recombinant strains of B. subtilis which are eligible for the TSCA section 5(h)(4) exemption present no unreasonable risk. While not completely innocuous, B. subtilis presents low risk of adverse effects to human health or the environment. Because of the change in classification, older industrial strains of B. subtilis may not meet the present-day designation. As part of their eligibility for this TSCA section 5(h)(4) exemption, companies are required to certify that they are using B. subtilis. It is therefore expected that companies will have information in their files which documents the correct identification of their strains. Additionally, it is expected that companies will choose well-characterized industrial strains for further development through genetic modification. These expectations in combination with the use of Good Laboratory Practices should ensure the use of the correct species.

Because the recipient microorganism was found to have little potential for adverse effects, introduced genetic material meeting the specified criteria would not likely significantly increase potential for adverse effects. As further assurance that risks would be low, EPA is specifying procedures for minimizing numbers of organisms emitted from the facility for the Tier I exemption and will be reviewing the conditions selected for the Tier II exemption.

Modification of the language of the two proposed containment requirements § 725.422 does not affect EPA's original determination that microorganisms that are eligible for and used under the conditions of the Tier I exemption will not present an unreasonable risk of injury to human health or the environment. Increased exposure to the microorganisms within or outside the facility resulting from these revisions will be minimal. The risk of using this microorganism in fermentation under the final conditions of this exemption is still low.

When balanced against resource savings for society and expected product benefits, this exemption will not present unreasonable risks.

VII. ACTION

Bacillus subtilis is included as a recipient microorganism at § 725.420 for the tiered exemption.

Attachment I - Final Risk Assessment of Bacillus subtilis

Note: For Attachment I to this Final Decision Document, see "Final Risk Assessment of Bacillus subtilis" appearing elsewhere in the list of "Support Documents."